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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

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Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation: Scotland

8531816001

4. Title of the invention

ASSAY FOR ACTIVATED FACTOR XII

5. Name of your agent *(if you have one)*

Abel & Imray

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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ASSAY FOR ACTIVATED FACTOR XII

INTRODUCTION

The present invention relates to Factor XII, a component of the "contact activation system".

BACKGROUND OF THE INVENTION

Factor XII is an inactive zymogen present in normal blood. It is readily converted, in vitro, in the presence of kallikrein, high molecular weight kininogen and a negatively charged surface into a form, Factor XIIa, that is enzymatically active. *In vitro*, two forms of XIIa have previously been reported. The 80Kd form of the serine proteinase, often called Factor αXIIa, has a 52Kd heavy chain linked by a disulphide bond to a 28Kd light chain. Proteolysis of this factor releases a peptide from the heavy chain, and results in a product, Factor βXIIa, that retains serine protease activity, but in which the 28Kd chain of Factor αXIIa is disulphide-linked to a small peptide fragment derived from the former 52-Kd heavy chain. In many cases the small peptide fragment has a molecular weight of about 1000d, but fragments of different size have been observed.

WO90/08835 discloses an immunoassay for Factor XIIa. WO 90/08835 also discloses monoclonal antibodies 2/215 and 201/9, which bind to Factor XIIa, and methods for their production. Monoclonal antibody (mAb) 2/215 is produced by hybridoma 2/215, deposited at the European Collection of Animal Cell Cultures, Divisional of Biologics, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury SP4 0JG, England (known as ECACC) on 16 January 1990 under the deposit number 90011606, and hybridoma 201/9, producing

monoclonal antibody 201/9, was deposited at ECACC on 18 January 1990 under deposit number 90012512.

Factor XIIa has long been known to be involved in the contact system of blood coagulation in vivo. More recent work indicates that Factor XIIa is also involved in other systems, including fibrinolysis, kininogensis, and also complement activation and angiogenesis. Many clinical and experimental data are accumulating to suggest that the contact system extends beyond haemocoagulation and that it has a role in maintaining vascular wholeness and blood pressure, that it influences various functions of endothelial cells and that it is involved in control of fibrinolysis and in maintaining the constitutive anticoagulant character of the intravascular space. Further clinical and experimental studies indicate that the contact system is involved in acute and chronic inflammation, shock of different aetiologies, diabetes, allergy, thrombo-haemorrhagic disorders including disseminated intravascular blood coagulation, and oncological diseases. Such conditions, include sepsis, spontaneous abortion and thromboembolism. In addition, Factor XIIa may be involved in tissue defence and repair. Yarovaya et al. (Yarovaya, G.A., Blokhina, T.B. & Neshkova,E.A. Contact system. New concepts on activation mechanisms and bioregulatory functions.Biochemistry (Mosc). 2002 Jan;67(1):13-24) is a recent review of the contact system and new concepts on activation mechanisms and bioregulatory functions.

SUMMARY OF THE INVENTION

The present invention is based on our surprising observation that activated Factor XII (Factor XIIa) is present in urine, and that measurement of this urinary Factor XIIa provides information relating to a variety of clinical conditions.

The present invention provides a method which comprises detecting or determining Factor XIIa in urine.

The present invention provides a monoclonal antibody that is capable of binding to urinary activated Factor XII, for example, a monoclonal antibody having the same or similar properties as mAb 2/215 in relation to binding to Factor XIIa, but not including mAb 2/215 itself.

The present invention also provides a method for producing a monoclonal antibody that binds urinary activated Factor XII, which comprises raising monoclonal antibodies against Factor XII or a fragment thereof and screening the antibodies against urinary activated Factor XII.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the protocol used to carry out an immunoassay for cell-bound Factor XIIa using the IMx system of Abbott Laboratories.

Figure 2 shows HPLC traces using fluorescence detection of a, urine sample only, b, FITC labeled 2/215 antibody, c, urine incubated with FITC labeled 2/215 antibody, d, trace c after subtraction of traces a and b.

Figure 3 shows radioactivity in urine incubated with radiolabelled 2/215 Fab Fragment, upon separation of components using HPLC. Peak 1 is the result of 2/215 Fab binding to plasma components, peak 2 is the remaining unbound 2/215 Fab.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method which comprises detecting or determining activated Factor XII in a urine sample obtained from a mammalian subject, generally a human.

The term "antibody" as used herein includes any antibody fragment that is capable of binding antigen, for example, Fab and $F(ab')_2$ fragments, and also recombinant, chimeric and humanized antibodies.

Methods of carrying out immunoassays are well known, see for example, Methods in Enzymology, H. Van Vunakis and J. J. Langone (Eds), 1981, 72(B); Practice and Theory of Enzyme Immunoassays, P Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology, R. J. Burden and P. H. Van Knippenberg (Eds), Elsevier, 1985; Introduction to Radioimmunoassay and Related Techniques, T. Chard, ibid, 3rd Edition, 1987; and Methods in Enzymology, H. Van Vunakis and J. J. Langone (Eds) 1981, 74(C).

Immunoassay techniques, both qualitative and quantitative, include ELISA (enzyme linked immunosorbent assays), Western blotting, fluid phase precipitation assays, coated particle assays, competitive assays, sandwich assays, including forward, reverse and simultaneous sandwich assays, and solid phase radio immunoassays (SPRIA).

In one ELISA format that may be used according to the present invention, a capture antibody, especially a monoclonal antibody, that is capable of binding to urinary activated Factor XII, is immobilized on a solid phase support, for example, on a plastic or other polymeric material, for example on the wells of plastic microtitre plates, or on

beads or particles, for example, as used in proprietary systems, for example, the IMx system of Abbott Laboratories, Abbott Park, Illinois USA. Samples comprising mammalian urine are incubated in contact with the immobilised capture antibody and any resulting captured activated Factor XII is detected using a labeled antibody that is also capable of binding to urinary activated Factor XII.

The labeled antibody may be polyclonal or monoclonal. Anti-human antibodies, for example, anti-human polyclonal antibodies, are often convenient for use as labeled antibodies. The label may be detectable directly or indirectly. Any appropriate radioisotope may be used as a directly detectable label, for example a β-emitter or a γ-emitter, examples being ^{125}I, ^{131}I, ^{3}H, and ^{14}C. For commercial use, non-radioactive labels, generally enzyme labels, are preferred. Enzyme labels are detectable indirectly. An enzyme label is, for example, alkaline phosphatase or a peroxidase, for example, horse radish peroxidase. An appropriate substrate for the chosen enzyme, for example, a substrate that gives rise to a detectable optical or fluorescence change, for example, phenolphthalein monophosphate or a fluorescent substrate, for example, methyl umbeliferone, is used. Alternatively, there may be used an enzyme reaction that can be followed using an electrochemical method.

Activated Factor XII, that is labeled, for example, radiolabelled or enzyme-labeled, may be used in a competitive assay for measurement of urinary activated Factor XII.

An example of an immunoassay for Factor XIIa is that described in WO90/08835. To determine urinary activated Factor XII it is recommended that mAb 2/215 is used,

especially as the capture antigen. A different antibody, for example, a polyclonal antibody or a different monoclonal antibody may be used for detection.

Further methods utilise direct detection of a resulting antibody-antigen complex. Examples of such techniques are Surface Plasmon Resonance, Surface Acoustic Wave and Quartz Crystal Microbalance methodologies (Suzuki M, Ozawa F, Sugimoto W, Aso S. Anal Bioanal Chem 372:301-4, 2002; Pearson JE, Kane JW, Petraki-Kallioti I, Gill A, Vadgama P. J Immunol Methods ;221:87-94, 1998; Weisch W, Klein C, von Schickfus M, Hunklinger S. Anal Chem 1996 68:2000-4, 1996; Chou SF, Hsu WL, Hwang JM, Chen CY. Clin Chem 48:913-8, 2002).

A standard suitable for an assay for detection or determination of urinary activated Factor XII would typically comprise of a solution containing a known amount of activated Factor XII.

The invention, especially the immunoassays described above, provides a method of determination of urinary activated Factor XII, that can be used readily on automated equipment for large scale use.

Monoclonal antibodies and immunoassays according to the present invention may be used in studies of coagulation systems and of thrombotic and other disorders, see also below.

The present invention further provides a kit for carrying out an immunoassay of the present invention, which kit comprises, each in a separate container or otherwise compartmentalised: (i) a monoclonal antibody that is capable of binding urinary activated Factor XII, for

example, mAb 2/215 or another monoclonal antibody having the same or similar Factor XIIa binding properties as mAb 2/215, and (ii) a labeled antibody capable of binding to urinary activated Factor XII when urinary activated Factor XII is bound to the monoclonal antibody defined in (i).

The kit may comprise further components for carrying out an immunoassay, for example, as described above. The monoclonal antibody may be immobilised on a solid support.

A kit according to the invention may comprise, for example,

a) a monoclonal antibody that is capable of binding to urinary activated Factor XII, for example, mAb 2/215 or another monoclonal antibody having the same or similar Factor XIIa binding properties as mAb 2/215,

(b) a standard typically comprising of a solution containing known amounts of activated Factor XII,

(c) labeled antibody capable of reacting with urinary activated Factor XII when urinary activated Factor XII is bound to the monoclonal antibody defined in (i).

Alternatively, a kit may comprise labeled activated Factor XII, for use in a competitive assay.

A kit may also comprise further components, each in a separate container, for example, diluent(s), wash reagent solution(s) and substrate solution(s).

Factor XII and its activated form, Factor XIIa, are involved in blood coagulation and other contact systems, also known as contact phase systems, for example, fibrin-olysis, complement cascade, inflammation and vasodilation, see Jacobsen S. and Kriz M., Br J Pharmacol., 29, 25-36, 1967; Kurachi K et al,

Biochemistry, 19, 1330-8 1980; Radcliffe R et al, Blood, 50, 611-7, 1977; Ghebrehiwet B et al, J Clin Invest,71, 1450-6. 1983; Z Toossi et al, Proc Natl Acad Sci USA, , 89, 11969-72, 1992; Wachtfogel YT et al, Blood 67, 1731-7, 1986; Wachtfogel YT et al, Thromb Haemost, 80, 686-91, 1998; and Schreiber et al AD, J Clin Invest. 52, 1402-9, 1973.

As Factor XII and its activated form, Factor XIIa are involved in haemocoagulation and have a role in maintaining vascular wholeness and blood pressure, in influencing various functions of endothelial cells, in control of fibrinolysis and in maintaining the constitutive anticoagulant character of the intravascular space, measurement of urinary activated Factor XII is useful in investigations of those systems, including for example, fibrinolysis, complement cascade, inflammation and vasodilation. Clinical and experimental studies indicate that the contact system, which includes Factor XIIa, is involved in acute and chronic inflammation, shock of different aetiologies, diabetes, allergy, thrombo-haemorrhagic disorders including disseminated intravascular blood coagulation, oncological diseases, cardiovascular conditions, for example, myocardial infarction, angina and acute coronary syndrome, angiogenesis, sepsis, spontaneous abortion and thromboembolism.

Determination of urinary activated Factor XII, are therefore useful in clinical and scientific investigations of such conditions, including diagnosing, predicting susceptibility to, monitoring and monitoring treatment of disorders where the contact system is involved, including acute and chronic inflammation, shock of different aetiologies, diabetes, allergy, thrombo-haemorrhagic disorders including disseminated intravascular blood coagulation and thromboembolism, oncological diseases, cardiovascular

conditions, for example, myocardial infarction, angina, acute coronary syndrome, angiogenesis, sepsis, spontaneous abortion. Determination of urinary activated Factor XII is also useful in clinical and scientific investigations of renal disease, where it acts as a sensitive marker of renal damage, particularly in renal diseases where extensive proteinuria is not present.

Detection or determination of urinary activated Factor XII, is therefore useful as an aid to diagnosing or monitoring diseases and disorders in which the amount of urinary activated Factor XII is different from that in healthy subjects. Changes in the level of urinary activated Factor XII may be indicative of any of the conditions mentioned above. Changes in level in a subject with time may be indicative of change in the condition, for example, exacerbation of the condition, or improvement, for example, in response to therapy. Such methods of diagnosis and monitoring are part of the present invention.

The present invention provides a monoclonal antibody that is capable of binding to urinary activated Factor XII, for example, a monoclonal antibody having the same or similar properties as mAb 2/215 in relation to binding to Factor XIIa, but not including mAb 2/215 itself.

A monoclonal antibody that is capable of binding to urinary activated Factor XII, for example, a monoclonal antibody having the same or similar properties as mAb 2/215 in relation to binding to Factor XIIa, may be produced by methods that are known per se. Resulting antibodies are screened for those having the desired characteristics.

It may be useful to use monoclonal antibody 2/215 as a reference antibody in the screens for antibodies that bind to urinary activated Factor XII. A selected antibody may have binding characteristics for urinary activated Factor XII that are the same as or similar to those of mAb 2/215.

The antigen used to raise the antibodies is Factor XII or a fragment thereof. An antigenic fragment of Factor XII may itself be immunogenic or may be too small to be immunogenic, in which case it may be converted into an immunogen, for example, by conjugation to another peptide, for example, as described below. The term "an antigenic fragment of Factor XII" as used herein includes both a fragment, for example, a peptide, and an immunogenic form of such a fragment if it is not itself immunogenic.

An antigenic fragment of Factor XII may be Factor XIIa, for example, Factor α-XII or Factor β-XIIa or a fragment thereof, for example, a peptide that is a fragment of Factor βXIIa that is or that includes at least one antigenic determinant capable of recognising anti-Factor βXIIa.

Methods of preparing immunogens are known to those in the art. Any of these methods may be utilised to render immunogenic or to improve the immunogenicity of Factor XII or antigenic fragment thereof, see also WO90/08835.

For example, Factor βXIIa may be used as the immunogen to raise anti-Factor XIIa monoclonal or polyclonal antibodies. Factor βXIIa may be produced by a method which comprises first isolating Factor XII from fresh or freshly frozen plasma, for example, using a combination of ammonium sulphate precipitation and anion exchange chromatography for example,

according to the method described by K. Fujikawa and E. W. Davie (Methods in Enzymol, 1981, 80, 198-211). Methods for converting Factor XII to Factor βXIIa and isolating Factor βXIIa from the resulting mixture are described by K. Fujikawa and B. A. McMullen (Journal of Biol.Chem., 1983, 258, 10924-10933) and B. A. McMullen and K. Fujikawa (Journal of Biol. Chem. 1985, 260, 5328). To obtain Factor βXIIa, Factor XII is generally subjected to limited cleavage, for example, by chemical or enzymatic digestion, for example, using trypsin or a trypsin-like enzyme, generally in a highly diluted form, for example, in a molar ratio of trypsin:Factor XII of 1:500, for example, in a weight ratio trypsin:Factor XII of 1:75 and the cleavage products separated, generally by chromatography.

An antigenic fragment of Factor βXIIa may be produced by degradation of Factor βXIIa by enzymatic or chemical means. For example the disulphide-linked light chain peptide of Factor βXIIa can be obtained by reduction and carboxymethylation of Factor βXIIa and isolation of the fragment by chromatography (K. Fujikawa and B. A. McMullen Journal of Biol. Chem. 1983, 258, 10924). Alternatively, an antigenic fragment of Factor βXIIa may be produced if its amino acid sequence is known, synthetically, as may Factor βXIIa itself. Any of the many known chemical methods of peptide synthesis may be used, especially those utilising automated apparatus.

An antigenic fragment of Factor βXIIa may be produced using the techniques of recombinant DNA technology, as may Factor βXIIa itself. Cool et al, 1985 and 1987, loc. cit. have characterised a human blood coagulation Factor XII cDNA and gene. Recombinant production may be achieved by known methods, see for example, WO90/08835.

Unless specified otherwise, the terms "Factor βXIIa" and "βXIIa" as used herein include antigenic fragments of the Factor βXIIa molecule.

A monoclonal antibody for use according to the present invention must be capable of binding urinary activated Factor XII.

It is preferable, that a monoclonal antibody for use according to the present invention shows no significant binding to Factor XII zymogen. In the latter case, the corrected cross-reactivity with Factor XII is, for example, 0.1% or less. A factor to take into consideration in assessing the cross-reactivity of an antibody of the invention with Factor XII is that even "pure" Factor XII preparations are almost inevitably contaminated with small amounts of Factor XIIa (Silverberg and Kaplan, Blood 60, 1982, 64-70). WO90/08835 gives details of methods of assessing the corrected cross-reactivity with Factor XII. Unless specified otherwise, the term "cross reactivity" is used herein to mean the corrected cross reactivity.

Methods used to produce monoclonal antibodies are well known, see for example, Methods in Enzymology, H. Van Vunakis and J. J. Longone (Eds) 1981, 72(B) and ibid, 1983 92(E).

Monoclonal antibodies may be produced, for example, by a modification of the method of Kohler and Milstein (G. Kohler and C. Milstein, Nature, 1975, 256, 495): female Balb/C or C57/BI0 mice are immunised by intraperitoneal injection of Factor XII or an antigenic fragment thereof, for example, from 10 to 30 μg, generally 20 μg of Factor βXIIa or a corresponding amount of the other antigen. The Factor βXIIa or other antigen is preferably conjugated to another protein

molecule, for example, to a purified protein derivative of tuberculin or, preferably, to bovine thyroglobulin. The conjugation may be carried out, for example, by a carbodiimide method or by using a hetero-bifunctional reagent. The immunogen is generally presented in an adjuvant, preferably complete Freunds adjuvant. This procedure is generally repeated at intervals, generally using the same immunogen in the same dose, for example, at 3 week intervals the mice are boosted with 20 μg of conjugated Factor βXIIa in complete Freunds adjuvant until suitable response levels are observed. A pre-fusion boost is preferably given prior to sacrifice, for example, intravenously 3 days prior to sacrifice.

The antibody response is monitored, for example, by RIA antisera curve analysis using, for example, ^{125}I radiolabelled Factor XII or a fragment thereof, for example, radiolabelled Factor βXIIa or another Factor βXIIa antigen prepared by the chloramine-T method (P. J. McConahey and F. J. Dixon, Int. Arch. Allergy Appl. Immunol, 1966, 29, 185). Purity is confirmed, for example, by using autoradiography, for example, of SDS-PAGE gels run under reducing conditions.

Immune mouse spleen cells are then fused with myeloma cells, for example, NSO mouse myeloma cells, for example in the presence of 40-50% PEG 4,000 or 50% PEG 1500. The cells are then seeded in wells of culture plates and grown on a selective medium. The supernatants are tested for reactivity against the corresponding purified Factor XII antigen, for example, in the case of a Factor βXIIa antigen, purified Factor βXIIa or other βXIIa antigen, for example, by a solid phase enzyme immunoassay, for example, using peroxidase-labeled anti-mouse IgG. All wells showing specificity for the antigen used for testing are generally taken for further

secondary screening. The secondary screening consists, for example, of screening all specific antibodies for binding in solution to the appropriate antigen, for example, in the case of a Factor βXIIa antigen, Factor βXIIa or a Factor βXIIa antigenic fragment that has been radiolabelled. These are preferably titrated to determine the antibody dilution required for 50% B max. Dose-response curves against cold, that is to say non-labeled antigen are generated, and are preferably also generated against Factor XII (if no cross-reactivity with Factor XII is desired), plasmin and fibronectin. The extent of cross reaction may be determined according to the following formula:

$$\frac{\text{Weight of Cold Standard Antigen to Achieve 50\% B max}}{\text{Weight of Cross-Reactant to achieve 50\% B max}} \times 100$$

Those antibodies showing an appropriate level of binding to the desired antigen, Factor βXIIa, for example, having affinity constants of at least $10^{10}M^{-1}$ are generally taken forward for cloning.

Successful clones are generally isotyped. The cells are then preferably sub-cloned by limiting dilution and again screened, generally using an enzyme immunoassay, for the production of antibodies to the desired antigen, for example Factor βXIIa. A selected sub-clone from each cloning may also be evaluated with respect to specificity and dose response using a radioimmunoassay or ELISA.

The antibodies may be screened for those showing a pre-determined apparent cross reactivity to Factor XII, preferably of 1.5% or less, for example 1% or less, for example 0.5% or less, for example, 0.1% or less.

As indicated above, screening against Factor XIIa is generally carried out first, but the two or optionally three screens may be carried out in any order.

Scatchard analysis may be done on the dose-response data to produce values for the affinity constants for each antibody.

Sub-cloned or cloned hybridoma cells may be injected intraperitoneally into Balb/C mice for the production of ascitic fluid. The immunoglobulin may be precipitated from ascitic fluid, for example, at 4°C using saturated ammonium sulphate solution (equal volume). The precipitate is preferably purified, for example, it may be centrifuged, dissolved, for example, in 50mM Tris-HCl buffer pH 7.5 (volume equal to original ascites volume) and then dialysed against the same buffer. The immunoglobulin fraction may then be further purified by anion exchange chromatography, for example, the protein solution may be applied to a Mono-Q anion exchange column (Pharmacia) and eluted using a salt gradient in the same buffer according to the manufacturer's recommendations. The fractions containing immunoglobulin are generally pooled and frozen at -20°C for storage. Alternatively, hybridoma cells may be grown in culture for antibody production and the antibody isolated essentially as described above for ascites fluid.

Although the hybridomas described herein are derived from mouse spleen cells, the invention is not limited to hybridomas of murine or part-murine origin. Both fusion partners (spleen cells and myelomas) may be obtained from any suitable animal. Recombinant antibodies may be produced. Antibodies may be brought into chimeric or humanized form, if desired. The hybridomas are preferably cultured in vitro.

The present invention also provides polyclonal antibodies, also called a polyclonal antiserum, that are capable of reacting with urinary activated Factor XII. Such antibodies may be labeled and used for detection of captured urinary activated Factor XII, in an ELISA.

The invention also provides a method for the production of such a polyclonal antiserum, which comprises administering Factor XII or a fragment thereof, for example, Factor XIIa, especially Factor βXIIa to an animal, obtaining serum from the animal, screening the serum for binding to urinary activated Factor XII.

The following non-limiting Examples illustrate the present invention.

EXAMPLES

Example 1.

Microtitre plate assay

5 normal random urine samples were obtained from healthy male volunteers. These samples were tested for the presence of activated Factor XII using a microtitre plate assay as described below.

100μl aliquots of sample were added to wells of a microtitre plate precoated with 2/215 Monoclonal antibody. After incubation for 60 minutes, the plates were washed with a borate buffered saline wash solution (pH 7.4). 100 μl of conjugate (alkaline phosphatase labelled sheep polyclonal antibody raised against human βXIIa) was added to each well, and the plate was incubated for a further 60 minutes. After washing the plate again, 100 μl of phenolphthalein phosphate

substrate was added. After a suitable incubation period, an alkaline Stop solution was added to inhibit further substrate conversion, and the absorbance was recorded at 550nm. XIIa concentrations in the samples were then calculated by comparison of sample absorbances to those obtained for aqueous samples containing known concentrations of βXIIa. The resultant urinary activated Factor XII concentrations are shown in Table 1.

Table 1. Activated Factor XII concentrations as assessed by a microtitre plate assay in random urine samples from healthy male volunteers.

Volunteer	XIIa ng/ml
1	0.9
2	1.6
3	1.8
4	1.8
5	1.0

Example 2

IMx assay

The Abbott IMx system is an automated immunoassay analyser designed to run assays using enzyme immunoassay and fluorescence polarisation immunoassay technologies.

The technique used in these Examples is microparticle enzyme immunoassay (MEIA). MEIA technology uses microparticles coated with a capture molecule (in this case an antibody) specific for the molecule being measured. The effective surface area of the microparticles and diffusion distance between analyte and solid phase result in improved assay

kinetics, permitting MEIA assays to be completed more rapidly than many other immunoassays. The microparticles along with the bound analyte are separated from the reaction mixture by binding irreversibly to the glass fibre matrix used in the MEIA reaction cell.

The reactants necessary for MEIA assays are

- Microparticles coated with a capture molecule (in this case monoclonal antibody 2/215)
- Alkaline phosphatase-labeled conjugate (in this case antibodies against activated Factor XII, either polyclonal antibodies or mAb 2/215)
- Fluorogenic substrate, 4-methylumbelliferyl phosphate (MUP)
- Reaction cell that contains a glass fibre matrix to which immune complex binds.

Other reagents such as a diluent and/or wash solution are also required.

The following is a description of the MEIA Reaction process.

1. The IMx system transfers sample and microparticles (coated with capture molecules) to the incubation well of the reaction cell. During an incubation period, analytes bind to the microparticles, creating an immune complex.
2. The IMx System transfers an aliquot of the immune complex to the inert glass fibre matrix of the reaction cell. The immune complex binds irreversibly to the glass fibre matrix. The IMx washes the matrix to remove unbound materials, and the immune complex is retained by the glass fibres whilst the excess reaction mixture flows rapidly through the large pores in the matrix.
3. The IMx system adds alkaline phosphatase labelled conjugate to the matrix. The conjugate binds to the

immune complex to complete the antibody-analyte-conjugate "sandwich". The IMx washes the matrix again.

4. The IMx system adds the flurogenic substrate 4-methylumbelliferyl phosphate (MUP) to the matrix. The conjugate catalyses the hydrolysis of 4-methyl-umbelliferyl phosphate (MUP) to 4-methylumbelli-ferone (MU)
5. The MEIA optics within the IMx instrument measure the rate at which the fluorescent product (MU) is generated on the glass fibre matrix. The rate at which MU is generated on the matrix is proportional to the concentration of the analyte in the test sample.

The protocol used for experiments described below is set out in Figure 1 of the accompanying drawings.

5 normal random urine samples were obtained from healthy male volunteers. These samples were tested for the presence of activated Factor XII using a microtitre plate assay as described above. The resultant urinary activated Factor XII concentrations are shown in Table 2.

Table 2. Activated Factor XII concentrations as assessed by an IMx assay in random urine samples from healthy male volunteers.

Volunteer	XIIa ng/ml
A	0.5
B	3.3
B	2.8
D	2.3
E	0.9

Example 3.

In this example the existence of urinary activated Factor XII was demonstrated by binding to fluorescently labelled antibody, and separating the antibody that had bound to activated Factor XII from unbound antibody on the basis of molecular weight using high performance liquid chromatography (HPLC).

Antibody 2/215 was labelled with Fluorescein Isothiocyanate (FITC)(Pierce, 3747 N Meridian Road, PO Box 117, Rockford, IL 61105. U.S.A.) as per manufacturer's instructions.

The HPLC system consisted of a Waters 1525 Binary HPLC Pump, a Waters 2487 Dual λ Absorbance Detector, and Jasco FP1520 Integral Fluorescence Detector.

The mobile phase used for the HPLC was 0.1M NaCl 0.05M Tris HCl, 0.4%(w/v) Tri-sodium citrate pH 7.5. The stationary phase comprised 2 x30 cm BioSep-SEC-S 3000 columns in series(Phenomenex, Queens Avenue, Hurdsfield Industrial Estate, Macclesfield, Cheshire SK10 2BN, United Kingdom). Flow rate was 1.0 ml min^{-1} and the injection volume was 100 μl. Settings for the Jasco Fluorescence detector were. Excitation wavelength 494nm, emission wavelength 520nm, Gain 1000, attenuation 1.

Samples run on the HPLC system were the FITC labelled 2/215 alone, a urine sample alone, and urine which had been incubated with FITC labelled 2/215 for 4 hours (250 μl urine plus 1 μl FITC labelled antibody).

Example plots of fluorescence versus time are shown in Figure 2.

In trace a) the urine sample alone it can be seen that the urine sample exhibits endogenous fluorescence. In trace b, fluorescence associated with the FITC labelled antibody is observed. In trace c), urine which has been preincubated with FITC labelled antibody a peak additional to those in Traces a and b is observed. This indicates that the FITC labelled antibody is binding to a component in the urine sample. This is further exhibited in Trace d) where the signals associated with endogenous fluorescence and the FITC labelled antibody alone have been subtracted, the resultant trace reflecting only the binding of the antibody to urinary activated Factor XII.

Example 4.

In this example the existence of urinary activated Factor XII in plasma was demonstrated by binding to antibody fragments labelled with a radiotracer (Iodine 125), and separating the resultant complexes on the basis of molecular weight using high performance liquid chromatography (HPLC).

The HPLC system consisted of an Agilent 1100 HPLC system.

Fab antibody Fragments of antibody 2/215 were prepared using an "Immunopure Fab Preparation Kit" (Pierce, 3747 N Meridian Road, PO Box 117, Rockford, IL 61105, U.S.A.) according to manufacturer's instructions. These Fab Fragments were then radiolabelled with Iodine 125 by Amersham Pharmacia Biotech (Pollards Wood, Nightingales Lane, Chalfont St Giles, HP8 4SP United Kingdom).

1 μl of radiolabelled antibody was added to 1ml of urine obtained from healthy volunteers. After incubation for 4

hours, the components of the plasma were separated by High Performance Liquid Chromatography (HPLC).

The mobile phase used for the HPLC was 0.1M NaCl 0.05M Tris HCl, 0.4%(w/v) Tri-sodium citrate pH 7.5. The stationary phase comprised 2 x30 cm BioSep-SEC-S 3000 columns in series (Phenomenex, Queens Avenue, Hurdsfield Industrial Estate, Macclesfield, Cheshire SK10 2BN, United Kingdom). Flow rate was 0.7 ml min^{-1} and the injection volume was 100 μl.

Fractions of the HPLC eluent were collected using an automated Fraction collector, set to collect one fraction every 20 seconds. Radioactivity was then measured in each fraction using a multiwell scintillation counter.

An example plot of radioactivity versus time is shown in Figure 3, where it can be seen that there is a peak additional to that of the unbound antibody fragment demonstrating that the radiolabelled antibody fragment has bound to activated Factor XII present in urine.

CLAIMS

1. A method which comprises detecting or determining activated Factor XII in a sample comprising urine obtained from a mammalian subject.

2. A method as claimed in claim 1 wherein a chromogenic assay is used to detect urinary activated Factor XII.

3. A method as claimed in claim 1, wherein an immunoassay is used to detect urinary activated XII.

4. A method as claimed in claim 3, wherein the sample is contacted with a labelled antibody that is capable of binding to urinary activated Factor XII and any resulting antigen-antibody complex is detected or determined.

5. A method as claimed in claim 3, wherein the antibody is mAb 2/215, which is produced by hybridoma 2/215, deposited at the European Collection of Animal Cell Cultures, Divisional of Biologics, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury SP4 0JG, England (known as ECACC) under the deposit number 90011606, or is another monoclonal antibody having the same or similar XIIa binding properties for urinary activated Factor XII as mAb 2/215.

6. A method of diagnosing or monitoring a disease or disorder in a subject, in which disease or disorder the amount of urinary activated Factor XII differ from those in a subject not having the disease or disorder, which comprises determining activated Factor XII in a sample comprising urine obtained from the subject under investigation.

7. A method as claimed in claim 6, which comprises comparing the level of urinary activated Factor XII with levels of urinary activated Factor XII in a sample obtained from a subject not having the disease or disorder.

8. A method as claimed in claim 6 or claim 7, wherein the disease or disorder is a disease or disorder of the coagulation system.

9. A method as claimed in claim 6 or claim 7, wherein the disease or disorder is associated with inflammation or the inflammatory response.

10. A method as claimed in claim 6 or claim 7, wherein the disease or disorder is sepsis.

11. A method as claimed in claim 6 or claim 7, wherein the disease or disorder is acute or chronic inflammation, shock of different aetiologies, diabetes, allergy, a thrombo-haemorrhagic disorder, an oncological diseases, or a cardiovascular condition.

12. A method as claimed in claim 6 or claim 7, wherein the disease or disorder is a myocardial infarction, acute coronary syndrome, angina, or thromboembolism

13. A method as claimed in claim 6 or claim 7, wherein the disease or disorder is spontaneous abortion.

14. A method as claimed in claim 6 or claim 7, wherein the disease or disorder results in renal damage.

ABSTRACT

ASSAY FOR ACTIVATED FACTOR XII

Detection or determination of urinary activated Factor XII are useful as an aid to diagnosing or monitoring diseases and disorders in which the amount of urinary Factor XIIa is different from that in healthy subjects. Changes in the level of urinary Factor XIIa may indicate, for example, changes in the coagulation system. Changes in level may be associates with inflammation or the inflammatory response.

15. A method as claimed in claim 6 or claim 7, wherein the acceptance or rejection of a renal transplant is monitored.

16. A method as claimed in any one of claims 6 to 15, wherein urinary activated Factor XII is determined by a method as claimed in any one of claims 1 to 5.

17. A monoclonal antibody having the same or similar activated urinary Factor XIIa binding properties as mAb 2/215, other than mAb 2/215.

18. A method for producing a monoclonal antibody that binds to urinary activated Factor XII which comprises raising monoclonal antibodies against Factor XII or a fragment thereof and screening the antibodies against urinary activated Factor XII

19. A method as claimed in claim 18, wherein Factor XIIa is used to raise the antibodies and are used for screening.

20. A method as claimed in claim 18 or claim 19, wherein mAb 2/215 is used as a reference antibody in screening.

60µl microparticles and 90µl sample added to reaction well

⇓

Reaction cell fibre matrix washed with 50µl prewash

⇓

Sample and microparticles incubated for 20 min.

⇓

100µl of 150µl sample/microparticle mix added to reaction cell fibre matrix

⇓

Reaction cell fibre matrix washed with 2 shots of 100µl MEIA buffer
(3 second delay between shots)

⇓

60µl of appropriate conjugate added to reaction cell fibre matrix

⇓

20min. incubation with conjugate on reaction cell fibre matrix

⇓

Reaction cell fibre matrix washed with 2 shots of 100µl MEIA buffer
(3 second delay between shots)

⇓

65µl MUP substrate added to Reaction cell fibre matrix

⇓

3 second delay then read

Figure 1

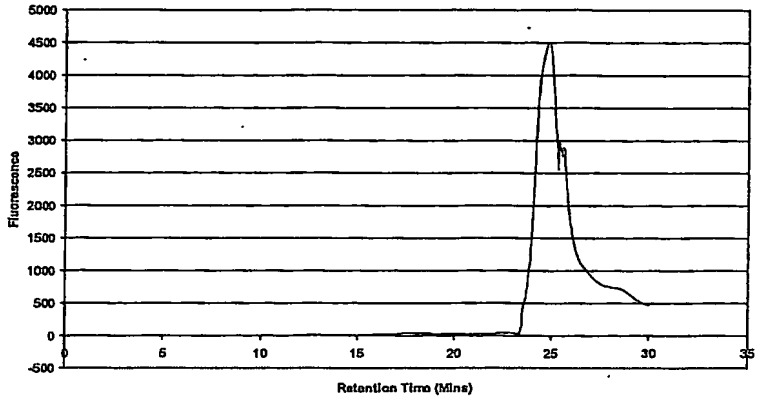

Figure 2a

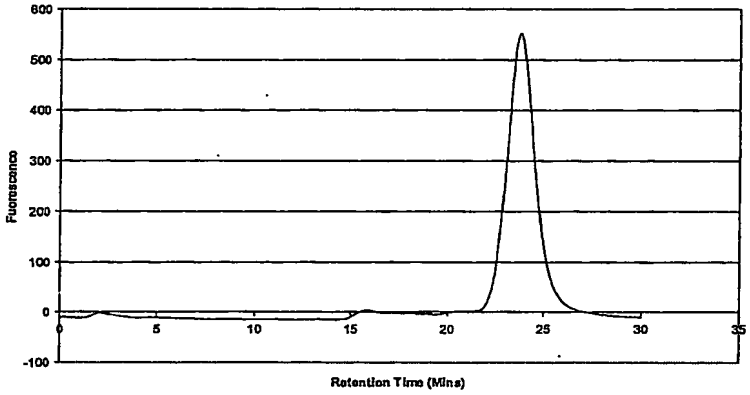

Figure 2b

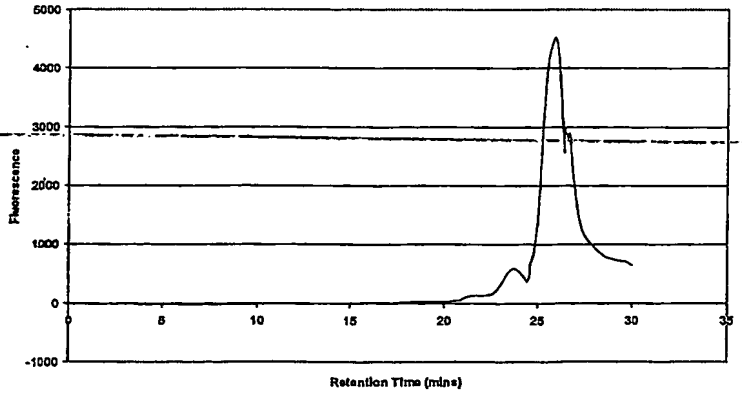

Figure 2c

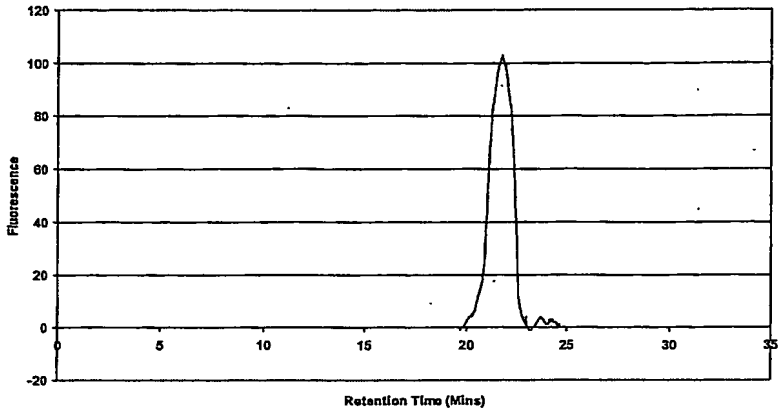

Figure 2d

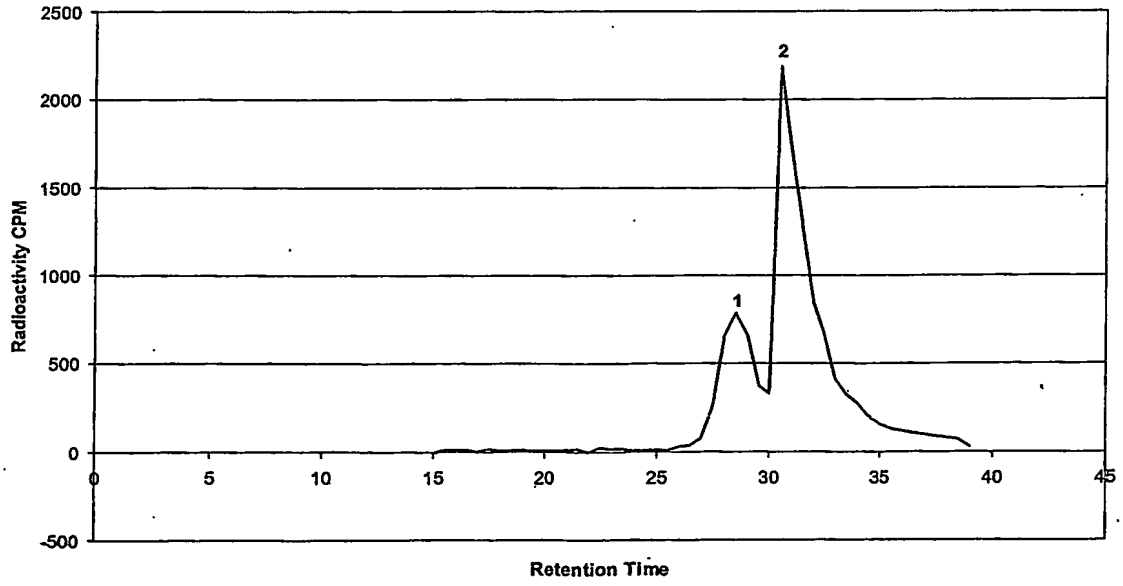

Figure 3